

African Journal of Biotechnology Vol. 10(71), pp. 15966-15972, 14 November, 2011
Available online at <http://www.academicjournals.org/AJB>
DOI: 10.5897/AJB11.1645
ISSN 1684-5315 © 2011 Academic Journals

Full Length Research Paper

Effect of lead on metallothionein concentration in lead-resistant bacteria *Bacillus cereus* isolated from industrial effluent

Shruti Murthy*, Geetha Bali and S. K. Sarangi

Department of Microbiology and Biotechnology, Bangalore University, Bangalore - 560056, Karnataka, India.

Accepted 5 September, 2011

Metallothioneins (MTs) are cysteine-rich metal-binding proteins found in a wide variety of organisms including bacteria, fungi as well as all eukaryotic plant and animal species. MTs bind essential and non-essential heavy metals. MTs production was evaluated by a simple spectrophotometry methodology. The study elucidated that the metallothionein concentration in *Bacillus cereus* treated with different concentrations of lead increased with increasing lead concentration. The protein samples from *B.cereus* treated with different concentrations of lead were isolated and electrophoresed on SDS-PAGE and 2-D gels. The study of gels revealed that a thick protein band appeared around 14.3KD in case of *B. cereus* treated with lead unlike the control samples.

Key words: *Bacillus cereus*, lead, metallothionein, industrial effluent.

INTRODUCTION

Excessive heavy-metal accumulation and circulation in the biosphere are important environmental and health concerns, due to the toxicity both in essential (Cu, Cr, Zn, Mn, Fe, Ni and Mo) and xenobiotic metals (Cd, Pb and Hg) at increased levels of bioavailability (Nriagu and Pacyna, 1988). Heavy metals have an important role in different biochemical reactions and are poisonous for cells in high concentrations (Nies, 1999). Unlike organic contaminants which can be converted into non-toxic derivatives, metals are intrinsically persistent in nature (Valls et al., 2000). Organisms respond to heavy metal stress using different defense systems such as exclusion, compartmentalization, formation of complexes and synthesis of binding proteins like MTs and phytochelatins (PCs). General toxicity mechanism for metal ions has divided into three categories: (1) blocking the essential biological functional groups of biomolecules especially proteins and enzymes, (2) displacing the essential metal ion in biomolecules, and (3) modifying the active

conformation of biomolecules resulting in the loss of specific activity. Microorganisms can affect heavy metal concentrations in the environment because they exhibit a strong ability for metal removal from solutions; this can be achieved either through enzymatic or non enzymatic mechanisms. Avoidance, restriction of metal entry into the cell, either by reduced uptake/active efflux or by the formation of complexes outside the cell and sequestration, reduction of free ions in the cytosol either by synthesis of legands to achieve intracellular chelation or by compartmentalization are the two major strategies of organisms to protect themselves against heavy metal toxicity.

Conventional technologies such as chemical precipitation, ion exchange or electrochemical process for removal of heavy metals are often neither effective nor economical, especially when used for the reduction of heavy metal ions to low concentrations (Wilde and Beneman, 1993). New separation methods are required to reduce heavy metal concentration to environmentally acceptable levels at affordable costs. Bio-removal has the potential to contribute to the achievement of this goal (Volesky, 1995). The uptake of metal occurs actively only within living cells through bioaccumulation or passively at the surface of both the living and the dead cells. This passive mechanism is called "biosorption". Filamentous

*Corresponding author. E-mail: murthy.shruti@gmail.com. Tel: 001-805-358-0410.

Abbreviations: MTs, Metallothioneins; PCs, phytochelatins; GSH, glutathione; PBS, phosphate buffer solution.

fungi, yeasts, bacteria and algae have proved to be good metal sorbents (Volesky, 1995). The removal of lead from contaminated wastewaters using bacteria (*Pseudomonas* sp., *Chryseomonas luteola* and *Bacillus circulans*) has been reported by several authors (Leung et al., 2000; Azza et al., 2009; Khanafari et al., 2008), who attempted to investigate the ability of lead biosorption by lead resistant bacteria isolated from an industrial effluent. The interest in the interaction of heavy metals with microorganisms has increased. The study of these interactions has focused in particular on the selection of metal resistant microorganisms from polluted environments (Hiroki, 1992; Kunito et al., 1997) and the possibility of using these microbes for detoxifying polluted environment (Wang et al., 1997; Taniguchi et al., 2000; Joseph et al., 2002).

Tolerance and removal of toxic metal ions have been studied in bacteria (Silver and Phung, 2005; Deveci et al., 2004; Yilmaz, 2003; Shakoori and Muneer, 2002; Cervantes et al., 2001; Ledin, 2000; Volesky and Holan, 1995), cyanobacteria (Wang et al., 2005; Inthorn et al., 1996), algae (Feng and Aldrich, 2004; Torricelli et al., 2004; Davis et al., 2003; Rehman and Shakoori, 2001), fungi (Pas et al., 2004; Yan and Viraraghavan, 2003; Holen and Volesky, 1995). Eccles (1995) has opined that the microbiological detoxification of polluted water is economical, safe and sustainable. Lead is a ubiquitous toxic metal which have mutagenic, carcinogenic, genotoxic, anthropogenic and phytotoxic effects (Alvarez et al., 2003; Zelikoff et al., 1988; Saenko et al., 1988). Sani et al. (2001) studied the effects of specific medium components on Pb toxicity to *Desulfovibrio desulfuricans* and toxicity was measured in terms of longer lag times and slower growth rates as compared to Pb-free controls. Dai et al. (2004) described the toxic effects of metals on C and N mineralization and microbial biomass. Metallothionein is a family of cysteine-rich, low molecular weight (MW ranging from 3500 to 14000 Da) proteins which have the capacity to bind both physiological (such as zinc, copper, selenium) and xenobiotic (such as cadmium, lead, mercury, silver and arsenic) heavy metals through the thiol group of its cysteine residues, which represents nearly the 30% of its amino acidic residues (Sigel, 2009). An exhaustive review has been done on metallothionein by various workers (Thirumoorthy et al., 2007; Mejare and Bulow, 2001; Hall, 2002; Silver and Ji, 1994). Although, the biological functions of MTs have not been fully elucidated, they are thought to play an important role in detoxification of toxic elements such as Cd^{2+} and Hg^{2+} . MTs also display antioxidant function, and are involved in Zn^{2+} homeostasis. These proteins can also be induced by a wide range of factors, such as hormones, physical stress and parasitism. They are present in a vast range of taxonomic groups, ranging from prokaryotes, protozoa, plants, yeast, invertebrates and vertebrates Pazirandeh et al. (1998); Neus et al. (2001), have studied the potential use of genetically-

engineered bacteria (expressing metallothionein) for water bioremediation. The objective of this research was to study metallothionein induction as a biomarker in the presence of a heavy metal (lead) by lead resistant bacteria *B. cereus* isolated from industrial effluent.

MATERIALS AND METHODS

Bacteria and maintenance

The bacterial species (*B. cereus*) used in this work was isolated from industrial effluent samples collected from Vrishabhavathi River flowing near Peenya Industrial area, Bangalore, Karnataka, India. Forty bacterial strains were isolated from the selected industrial effluents, on the basis of their ability to uptake lead. All the bacterial strains were subjected to metal tolerance studies and it was found that one bacterial strain was able to tolerate high concentrations of lead. This bacterial strain was isolated, purified and identified as *B. cereus* at Bangalore Genei, Bangalore. *B. Cereus* was maintained on trypton glucose extract agar.

Extraction and estimation of metallothionein

Trypton glucose extract broth supplemented with six different metal concentrations (0, 100, 200, 300, 400 and 500 mg/l) of $\text{Pb}(\text{NO}_3)_2$ was inoculated with 1ml of *B.cereus* culture and incubated on orbital shaker at 37°C for 48 h. After 48 h, all the six cultures were centrifuged at 10,000×g for 20 min and the pellets were dissolved in lysis buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, containing 0.01% mercaptoethanol). The pellets were sonicated for a total of 2 min (4 times for 30 s each time with a gap of 30 s between successive sonications). The sonicated sample was centrifuged at 30,000×g for 20 min to obtain a supernatant containing metallothionein. 1.05 ml of cold (-20°C) absolute ethanol and 80 µl of chloroform were added per 1 ml of the resulting supernatant. The samples were centrifuged cold (at 0–4°C) at 6000×g for 10 min and 3 volumes of cold ethanol was added to the resulting supernatant and stored at -20°C for 1 h. Samples were again centrifuged at 6000×g for 10 min. the resulting pellets were washed with ethanol: chloroform: homogenization buffer (87:1:12) and then centrifuged again at 6000×g for 10 min. The pellet was dried under a nitrogen gas stream to complete evaporation and the dried pellet was resuspended in 300 µl of 5 mM Tris-HCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 7. The resuspended MTs fraction was added to 4.2 ml of 0.43 mM 5, 5'-dithiobis (nitrobenzoic acid) in 0.2 M phosphate buffer, pH 8 and left at room temperature for 30 min. Then the concentration of reduced sulfhydryl was evaluated by reading the absorbance at 412 nm in a spectrophotometer. A standard curve with glutathione (GSH) was used as a standard reference for a correct quantification of MT in the samples. GSH contains one cysteine per molecule; thus, it is a standard for quantifying cysteines in protein analyses. Solutions containing different concentrations of GSH were prepared and their absorbance was measured at 412 nm. The amounts of metallothionein in the samples were estimated using the GSH standard, assuming that 1 mol of MT contains 20 mol of cysteine. For example, from this standard curve, if they find an absorbance of 2.5 in a sample, the x value is 0.44 (from the curve equation $x = (2.5 - 0.0524)/5.5553$). Then the MT content will be $0.44/20 = 0.022$ µmol. (Ana and Garcia-Vazquez, 2006)

Protein isolation and analysis

In order to quantify the protein concentration and to qualitatively

Table 1. The amounts of metallothionein in the samples estimated using the GSH standard.

Sample	Absorbance (412 nm)	x value [$x = \frac{\text{Absorbance} - 0.0524}{5.5553}$]
Control	0.00	-
100 mg/l	0.11 ± 0.07	0.007
200 mg/l	0.16 ± 0.02	0.016
300 mg/l	0.33 ± 0.04	0.047
400 mg/l	0.47 ± 0.02	0.072
500 mg/l	0.76 ± 0.06	0.125

Table 2. Metallothionein content in *Bacillus cereus* treated with different concentrations of lead.

Sample	MT Content (μmol) [x value / 20]
100 mg/l	35×10^{-5}
200 mg/l	8×10^{-5}
300 mg/l	24×10^{-4}
400 mg/l	36×10^{-4}
500 mg/l	63×10^{-4}

detect the expression, if any, of metal binding proteins, the following experiments were carried out. The selected isolate was grown in the medium amended with lead (100, 200, 300, 400 and 500 mg/l) with suitable control with no metal solutions added to the medium. 3 ml of the broth culture was removed from each flask and centrifuged at 10,000 rpm at 4°C for 5 to 10 min. The supernatant was discarded and 1 ml lysis buffer was added to the pellet. The pellet was sonicated for a total of 2 min (4 times for 30 s each time with a gap of 30 s between successive sonications). The sonicated sample was centrifuged at 5000 rpm at 4°C for 2 min. Two hundred microlitres of this supernatant was incubated with 1600 microlitres of acetone (always stored at -20°C) for 30 min at 4°C. The precipitated protein was dried under the clean bench for nearly 20 min. Five hundred microlitres of phosphate buffer solution (PBS) was added to this precipitated protein in order to form solution. Equal amount of protein samples were loaded and 12% SDS-PAGE was run using 14.3-97.4 KDa protein marker. The gels were stained overnight in Coomassie Brilliant Blue R-250, fixed in 10% acetic acid and destained in distilled water prior to scanning for documentation. 2 D gel electrophoresis was carried out for sample treated with 500 mg/l lead at Bangalore genei, Bangalore.

RESULTS

Estimation of metallothionein induction by lead on *B. cereus*

In order to estimate the amount of MT, the standard curve with GSH was used as a standard reference for a correct quantification of MT in the samples. GSH contains one cysteine per molecule; thus, it is a standard for quantifying cysteines in protein analyses. The amounts of

MTs in the samples were estimated using the GSH standard (Table 1) the estimation of MT concentrations in *B. cereus* is given in Table 2. The MT concentration in the control was zero whereas MT concentration in lead-supplemented samples (100, 200, 300, 400 and 500 mg/l) were 35×10^{-5} , 8×10^{-5} , 24×10^{-4} , 36×10^{-4} and 63×10^{-4} , respectively. A sympathetic relationship could be established between the concentration of Lead in the samples and their MT content. Overall, the MT concentrations in *B. cereus* were affected by lead supplementation.

Protein analysis

Figure 1 displays SDS-PAGE protein profile of *B. cereus* showing its response to different concentrations of lead (100, 200, 300, 400 and 500 mg/l). Interesting results were obtained based on the protein analysis of *B. cereus*. SDS-PAGE of *B. cereus* (control and lead supplemented samples) produced Coomassie blue staining protein patterns containing many distinct bands (Figure 1). The components ranged in molecular weight ranging from 14.3-97.4 KDa in 12% homogeneous separating gel. Significantly, one distinct band of low molecular weight protein (between 20.1 KDa and 14 KDa shown in arrow) was seen in lead supplemented samples but not in control sample. Figure 2 depicts the two dimensional gel electrophoresis of control and lead (500 mg/l) supplemented sample that was carried out to confirm the low molecular weight protein observed in SDS-PAGE. The presence of some over expressed distinct protein spots below 14.3 KDa (shown in arrow) in case of lead treated sample compared to control sample was confirmed as shown in the figure.

DISCUSSION

Environmental pollution due to discharge of untreated industrial effluents into water bodies has become a major concern in the developing countries today. Specially, the presence of toxic heavy metals contaminated in aqueous streams, arising from the discharge of untreated metal containing industrial effluents into water bodies, is viewed as one of the most important environmental issues. Although, investigations on effective remediation technologies have been carried out for many years, successful applications are limited (Volesky and Holan, 1995; Bailey et al., 1999). It is observed that the conventional technologies such as chemical precipitation, ion exchange or electrochemical process for removal of heavy metals are often neither effective nor economical, especially when used for the reduction of heavy metal ions to low concentrations (Wilde and Beneman, 1993). In order to overcome the ineffectiveness of the conventional technologies, new separation methods are required that

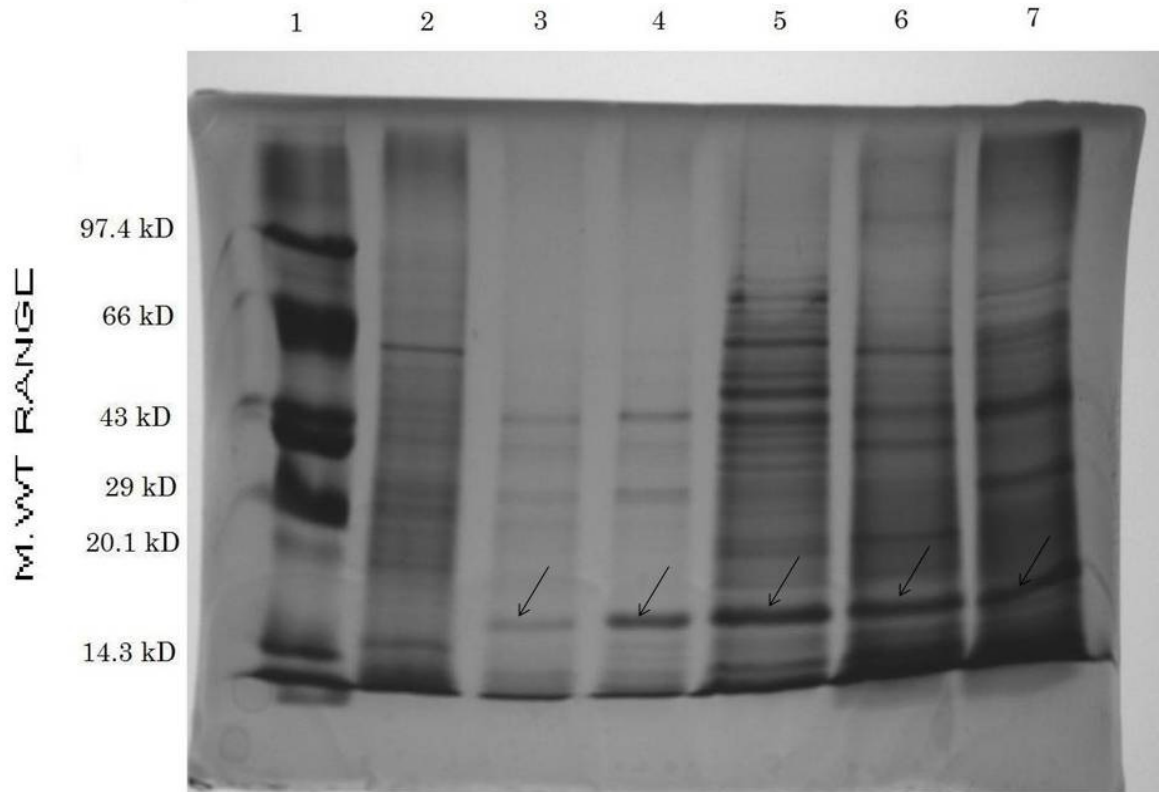


Figure 1. SDS-PAGE protein profile of *Bacillus cereus* showing its response to lead. From left to right; lane 1: 14.3-94.7 kD marker, lane 2: control, lane 3: 100 mg/l Pb, lane 4: 200 mg/l Pb, lane 5: 300 mg/l Pb, lane 6: 400 mg/l Pb and lane 7: 500 mg/l Pb. Arrows indicate MT.

reduce heavy metal concentration to environmentally acceptable levels at affordable costs. One of such method is bioremediation. Bio-removal has the potential to contribute to the achievement of this goal (Volesky, 1995). In recent years, the biosorption process has been studied extensively using microbial biomass as biosorbents for heavy metal removal. The metal removal abilities of various species of bacteria, algae, fungi and yeasts were investigated (Chen and Yiacoumi, 1997; Guibal et al., 1992; Veglio and Beolchini, 1997; Veglio et al., 1998; Utigikar et al., 2000; Gadd, 1992; Landgrave, 1995). Biosorption of heavy metals by microbial cells has been recognized as a potential, cost effective alternative to existing technologies for recovery of heavy metals from industrial waste streams (Gopal et al., 2002). Many aquatic micro-organisms, such as bacteria, yeast and algae can take up dissolved metals from their surroundings onto their bodies and can be used for removing heavy metal ions successfully (Asku et al., 1991).

One emerging strategy that is receiving more attention is the use of metalbinding peptides. Naturally occurring metal-binding peptides, such as MTs and polychelatins (Winklemann and Winge, 1994), are the main metal-sequestering molecules used by cells to immobilise metal ions, offering selective, high affinity binding sites. Over

expression of metal-binding proteins such as MTs in bacterial and yeast cells resulted in enhanced metal accumulation and thus offer promising strategy for the development of microbe-based biosorbents (Strouhal et al., 2003; Pazirandeh et al., 1995; Romeyer et al., 1990) for the removal and recovery of metals from contaminated water or soil. MTs have been found throughout the animal kingdom, in higher plants, in eukaryotic microorganisms, and in many prokaryotes. MTs have been divided into three classes. Based on their structural similarities: class I, II and III with multiple isoforms within each class. MT-I and MT-II are ubiquitously expressed and are stress inducible (Thirumoorthy et al., 2007). The amino acid sequences of MTs from many mammalian sources reveal that all contain approximately 61 amino acids of remarkably similar composition. More important, all contain 20 cysteine residues that remain invariant along the amino acid sequence. All cysteines are known to participate in the coordination of 7 mol of Cd or zinc (Zn) per mol of MT (5). Coordination of these cysteine residues results in a high binding affinity for Zn (10^{18}) and Cd (10^{22}) (Curtis et al., 1999). It is established from the current studies that there is a significant increase of MT biosynthesis in *B. cereus* after exposure to lead (100, 200, 300, 400 and 500 mg/l). It is significant to note

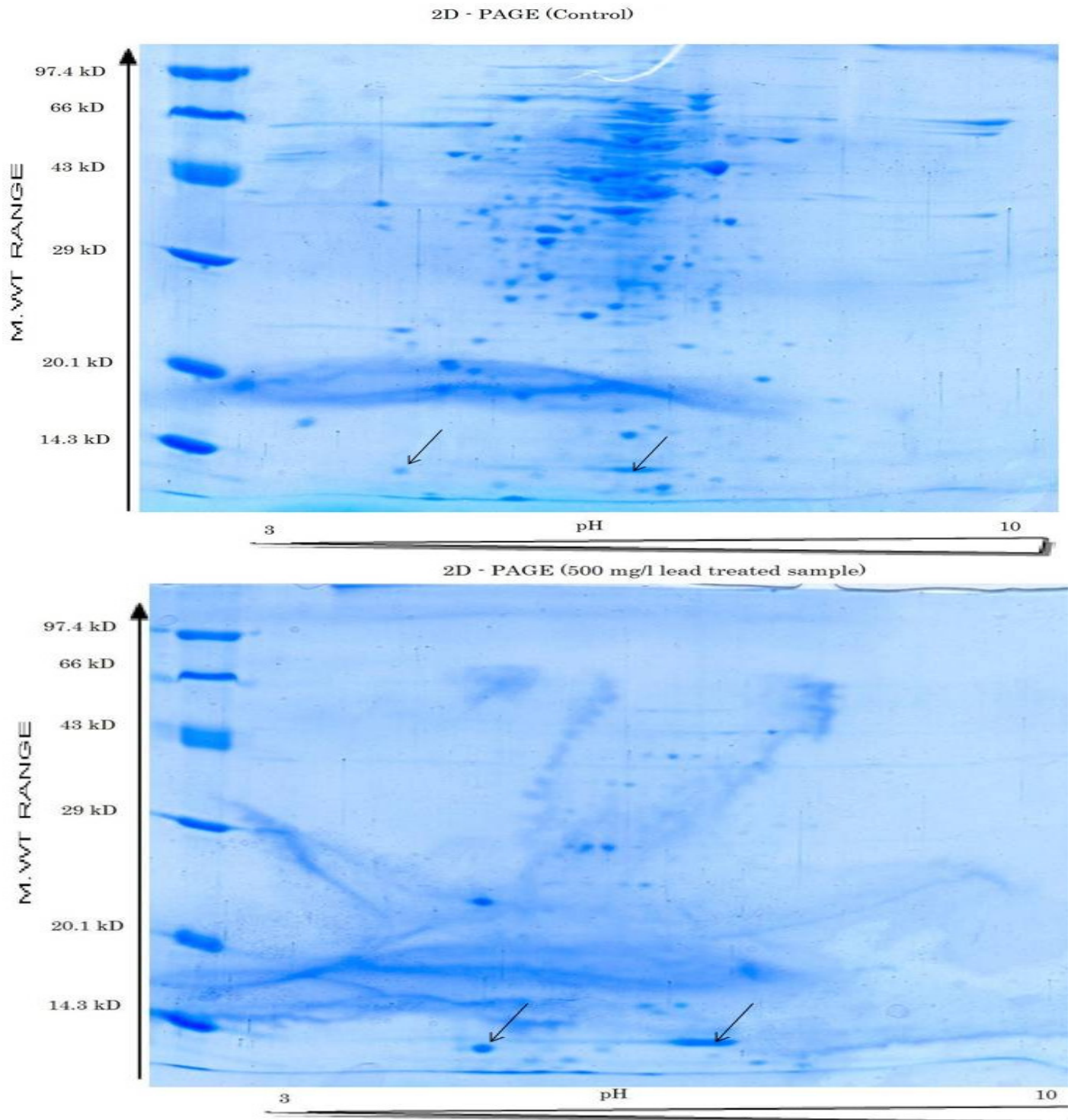


Figure 2. Two dimensional gel electrophoresis of *Bacillus cereus* protein (arrows indicate MT).

that though there exists a concomitant relationship between the concentration of lead in the samples and their MT content, a low MT content in the sample treated with 200 mg/l Pb is quite intriguing.

This could prove either the intake of Pb by *B. cereus* at

such a concentration is minimum or the Pb intake might also depend on other parameters such as pH and temperature conditions (which needs further studies to establish). The present study have further revealed that the MT production was induced in presence of lead and

hence it could be recommended as a biomarker in exposing lead.

Ryan and Hightower (1994) have postulated that all organisms from microbes to humans respond to chemical and physical stresses by increasing the synthesis of small group of stress proteins. One of the strategies of microbes against stress is retention or introduction of certain proteins, although, inhibition of proteins was also observed. In the present study, more protein bands were seen in lead treated samples as compared to control (Figure 1). However, it was observed that the protein bands were present in lead treated as well as in control. Shakoori et al. (2001) reported that the introduction of several new bands in the yeast isolates growing in M9-medium containing endosulfan shows extensive induction of several genes which are probably necessary for degradation of insecticide as well as adaptation of stressful conditions. MT constitute a class of low molecular weight, cystein rich metal binding proteins which are biosynthetically regulated at the level of gene transcription in response to heavy metal, hormones, cytokines, physiological and environmental stress (Zhou and Thiele, 1993; Tamai et al., 1994; Radtke et al., 1995; Liu and Thiele, 1996). SDS-PAGE pattern showed that in the case of treated sample some protein bands were over expressed compared to control.

It implicates the role of those protein bands in detoxification of metal stress. The SDS-PAGE analysis data implicated the induction or suppression of certain proteins. The expanding field of biotechnology helped to strengthen the idea that microbial enzymes could be used to control the fate of heavy metals in the environment. These isolates may be helpful in enzymatic bioremediation of polluted sites. They may provide a good source of xenobiotic degrading enzymes. The optimum growth conditions (pH and temperature) and growth curves provided base line data for bulk production of isolates and their use in bioremediation processes. Locally, isolated strains are beneficial in two ways. First, the local strains are better adapted to the environmental conditions, which have been prevalent for very long period. Secondly, the variation in genes and genetic elements acquired in response to certain selection pressures or due to spontaneous mutations enhance the survival values of microorganisms and provide opportunity for efficient exploitation in environmental cleanup operations. Microbes and the environment constantly interact with each other constitute a very ingenious system of evolving each other. Microbes are now at the forefront of the campaign against environment pollution due to their catabolic pathways. The properties of the bacterial strains confirmed their place as strong candidates for biosorption of lead. Moreover, these isolates could efficiently work in a microbe based reactor. They provide a base line data for future studies in this field. These bacterial strains can be further investigated for identification of particular gene and enzymes respon-

sible for bioremediation of lead.

ACKNOWLEDGMENTS

This work was financially supported by ISRO, Department of Space (DOS), Government of India. The authors thank the DOS for the JRF and also the Department of Microbiology and Biotechnology, Bangalore University, Bangalore for providing laboratory facilities.

REFERENCES

- Ana RL, Garcia-Vazquez E (2006). A simple assay to quantify metallothionein helps to learn about bioindicators and environmental health. *Biochem. Mol. Biol. Edu.* 34(5): 360–363.
- Alvarez E, Fernandez Marcos MI, Vaamonde C, Fernandez Sanjurjo ML (2003). Heavy metals in the dump of an abandoned mine in Galicia (NW Spain) and in the spontaneously occurring vegetation. *Sci. Total Environ.* 313: 185-197.
- Asku Z, Kutsal T, Gun S, Haciosmanoglu N, Gholmenejad M (1991). Investigation of biosorption of Cu (II), Ni (II) and Cr (VI) ions to activated sludge bacteria. *Environ. Technol.* 12: 915-921.
- Azza AAZ, Wesam AH, Hedayat MS, Ghada AF (2009). Biosorption of Some Heavy Metal Ions Using Bacterial Species Isolated from Agriculture Waste Water Drains in Egypt. *J. Appl. Sci. Res.* 5(4): 372-383.
- Bailey SE, Olin TJ, Bricka RM, Adrian DD (1999). A review of potentially low-cost sorbents for heavy metals. *Water Res.* 33(11): 2469-2479.
- Cervantes C, Campos-Garcia J, Devars S, Gutierrez-Corona F, Iloz-Tavera H, Torres-Guzman JC, Moreno-Sanchez R (2001). Interactions of chromium with microorganisms and plants. *FEMS Microbiol. Rev.* 25: 335-347.
- Chen J, Giacoumi S (1997). Biosorption of metal ions from aqueous solutions. *Separ. Sci Technol.* 32(1-4): 51-69.
- Curtis DK, Jie L, Supratim C (1999). Metallothionein: An intracellular protein to protect against cadmium toxicity. *Annu. Rev. Pharmacol. Toxicol.* 39: 267-294.
- Dai J, Becquer T, Rouiller JH, Reversat G, Bernhard RF, Lavelle P (2004). Influence of heavy metals on C and N mineralization and microbial biomass in Zn, Pb, Cu and Cd contaminated soils. *Appl. Soil Ecol.* 25: 99-109.
- Davis TA, Volesky B, Mucci A (2003). A review of the biochemistry of heavy metal biosorption by brown algae. *Water Res.* 37: 4311-4330.
- Deveci H, Akcil A, Alp I (2004). Bioleaching of complex zinc sulphides using mesophilic and thermophilic bacteria: comparative importance of pH and iron. *Hydrometallurgy.* 73: 293-303.
- Eccles H (1995). Removal of heavy metals from effluent streams. Why select a biological process? *Int. Biodeter. Biodegr.* 35:5-16.
- Feng D, Aldrich C (2004). Adsorption of heavy metals by biomaterials derived from the marine alga *Ecklonia maxima*. *Hydrometallurgy*, 73: 1-10.
- Gadd GM (1992). Metals and microorganisms: a problem of definition. *FEMS. Microbiol. Lett.* 79: 197-203.
- Gopal M, Pakshirajan K, Swaminathan T (2002). Heavy metal removal by biosorption using *Phanerochaete chrysosporium*. *Appl. Biochem. Biotechnol.* 102-103: 227-237.
- Guibal E, Roulph C, Le Cloirec P (1992). Uranium biosorption by a filamentous fungus *Mucor miehei* pH effect on mechanisms and performances of uptake. *Water Res.* 26(8): 1139-1145.
- Hall JL (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.* 53: 1-11.
- Hiroki M (1992). Effects of heavy metal contamination on soil microbial population. *Soil Sci. Plant Nutr.* 38: 141-147.
- Holan ZR, Volesky B (1995). Accumulation of cadmium, lead and nickel by fungal and wood biosorbents. *Appl. Biochem. Biotechnol.* 53: 133-146.

- Inthorn D, Nagase H, Isaji Y, Hirata K, Miyamoto K (1996). Removal of Cd from aqueous solution by the filamentous cyanobacterium *Tolypothrix tennisi*. J. Ferment. Bioeng. 82: 580-584.
- Joseph G, Swades L, Chaudhuri K, Kelly SD, Kemner KM, Oconnor SM, Coates JD (2002). Immobilization of radionuclides and heavy metals through anaerobic bio-oxidation of Fe (II). Appl. Environ. Microbiol. 68(6): 2704-2710.
- Khanafari A, Eshghdoost S, Mashinchian A (2008). Removal of lead and chromium from aqueous solution by *Bacillus circulans* biofilm. Iran. J. Environ. Health Sci. Eng. 5(3):195-200.
- Kunito T, Shibata S, Matsumoto S, Oyaizu H (1997). Zinc resistance of *Methylobacterium* sp. Biosci. Biotech. Biochem. 61: 729-731.
- Landgrave J (1995). A pilot plant for removing chromium from residual water of tanneries. Environ. Health Persp. 103(1): 63-65.
- Ledin M (2000). Accumulation of metals by microorganisms processes and importance for soil systems. Earth Sci. Rev., 51: 1-31.
- Leung WC, Wong MF, Chua H, Co W, Yu PHF, Leung CK (2000). Removal and recovery of heavy metals by bacteria isolated from activated sludge treating industrial effluents and municipal wastewater. Water sci. Technol. 41(12): 233-240.
- Liu XD, Thiele DJ (1996). Oxidative stress induced heat shock factor phosphorylation an HSF dependent activation of yeast metallothionein gene transcription. Gene Dev. 10: 592-603.
- Mejare M, Bulow L (2001). Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends Biotechnol. 19(2): 67-73.
- Neus C, Kirstine R, Roser G-D, Sílvia A (2001). Secretion of mouse-metallothionein by engineered *E. coli* cells in metal-enriched culture media, 3(4): 507-512.
- Nriagu JO, Pacyna JM (1988). Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature, 333: 134-139.
- Pas M, Milacic R, Draslar K, Pollak N, Raspor P (2004). Uptake of chromium (III) and chromium (VI) compounds in the yeast cell structure. Biometals, 17: 25-33.
- Pazirandeh M, Chrisey LA, Mauro JM, Campbell JR, Gaber BP (1995). Expression of the *Neurospora crassa* metallothionein gene in *Escherichia coli* and its effect on heavy metal uptake. Appl. Microbiol. Biotech. 43: 1112-1117.
- Pazirandeh M, Bridget MW, Rebecca LR (1998). Development of bacterium based heavy metal biosorbents: Enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. Appl. Environ. Microbiol. 64(10): 4068-4072.
- Radtke F, Georgive Q, Mucller RHP, Brucnera E, Schaffner W (1995). Functional domains of the heavy metal responsive transcription regulator MTF1. Nucleic Acids Res. 23: 2277-2286.
- Rehman A, Shakoori AR (2001). Heavy metal resistant *Chlorella* sp. Isolated from tannery effluents and their role in remediation of hexavalent chromium in industrial wastewater. Bull. Environ. Contamin. Toxicol. 66: 542-547.
- Romeyer FM, Jacobs FA, Brousseau R (1990). Expression of a *Neurospora crassa* metallothionein and its variants in *Escherichia coli*. Appl. Environ. Microbiol. 56: 2748-2754.
- Ryan JA, Hightower LE (1994). Evaluation of heavy metal ion toxicity in fish cells using a combined stress and cytotoxicity assay. Environ. Toxicol. Chem. 113: 115-120.
- Saenko GL, Zorina LG, Radkovich RD, Biryukova TA, Karyvin AV (1988). Trace elements in ecosystem of Peter the Great Bay of USSR. Okeanologiya, 28: p. 330.
- Sani RK, Geesey G, Peyton BM (2001). Assessment of lead toxicity to *Desulfovibrio desulfuricans* G20: influence of components of lactate C medium. Adv. Environ. Res. 5: 269-276.
- Shakoori AR, Qureshi AT, Saqib ZA (2001b). Endosulfan degrading yeast isolated from food industry effluents and their role in environmental cleanup. Proc. Pakistan Congr. Zool. 21: 69-94.
- Shakoori AR, Muneer B (2002). Copper resistant bacteria from industrial effluents and their role in remediation of heavy metals in wastewater. Folia Microbiol. 47: 43-50.
- Sigel A, Sigel H, Sigel RKO (2009). Metallothioneins and Related Chelators. Metal Ions in Life Sciences. 5. Cambridge: RSC Publishing. ISBN, 978-1-84755-899-2.
- Silver S, Phung LT (2005). A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. J. Ind. Microbiol. Biotechnol. 32: 587-605.
- Silver S, Ji G (1994). Newer Systems for Bacterial Resistances to Toxic Heavy Metals. Environ. Health Perspect. 102(3): 107-113.
- Strouhal M, Kizek R, Vacek J, Trnkova L, Nemecek M (2003). Electrochemical study of heavy metals and metallothionein in yeast *Yarrowia lipolytica*. Bioelectrochemistry, 60: 29-36.
- Tamai KT, Liu X, Silor P, Sosinowski T, Thiele DJ (1994). Heat shock transcription factor activates yeast metallothioneins gene expression in response to heat and glucose starvation via distinct signaling pathways. Mol. Cell. Biol. 14: 8155-8165.
- Taniguchi F, Suzuki Y, Kurihara H, Kurihara Y, Kasai H, Shirato S, Araie M (2000). Molecular cloning of the bovine MYOC and induction of its expression in trabecular meshwork cells. Invest. Ophthalmol. Vis. Sci. 41: 2070-2075.
- Thirumoorthy N, Manisenthil KKT, Shyam SA, Panayappan L, Malay C (2007). Metallothionein: An overview. World J. Gastroenterol. 13(7): 993-996.
- Torricelli E, Gorbi G, Pawlik-Skowronnska B, Sanita DL, Corradi MG (2004). Cadmium tolerance cysteine and thiol peptide levels in wild type and chromium-tolerant strains of *Scenedesmus acutus* (Chlorophyceae). Aquat. Toxicol. 68: 315-323.
- Utigikar VP, Chen BY, Tabak HH, Bishop DF, Govind R (2000). Treatment of acid mine drainage: I. Equilibrium biosorption of zinc and copper on non-viable activated sludge. Int. Biodeterior. Biodegrad. 46: 19-28.
- Valls M, Victor DEL, Roser GD, Sílvia A (2000). Engineering outer-membrane proteins in *Pseudomonas putida* for enhanced heavy – metal bioadsorption. J. Inorg. Biochem. 79: 219-223.
- Veglio F, Beolchini F (1997). Removal of metals by biosorption: a review. Hydrometallurgy. 44(3): 301-316.
- Veglio F, Beolchini F, Toro L (1998). Kinetic modeling of copper biosorption by immobilized biomass. Ind. Eng.Chem.Res. 37: 1107-1111.
- Volesky B, Holan Z R (1995). Biosorption of heavy metals. Biotechnol. Prog. 11: 235-250.
- Wang Z, Lu P, Liu D (1997). Initial assessment of the effects of rare earth elements on soil integrity. In: Proc. of the 2nd Sino-Dutch workshop on the Environ. Behavior and Ecotoxicology of Rare Earth Elements and Heavy Metals (eds. Guicherit R and Zhu W): TNO Publ., La Delft, the Netherlands. pp. 1-5.
- Wang WX, Del RC, Hong H (2005). Seasonal study on the Cd, Se and Zn uptake by natural coastal phytoplankton assemblages. Environ. Toxicol. Chem. 24: 161-169.
- Wilde EW, Beneman JR (1993). Bioremoval of heavy metals by use of microalgae. Biotech. Adv. 11: 781-812.
- Winklemann G, Winge DR (1994). Metal ions in fungi. Marcel Dekker, Inc., New York, N.Y.
- Yan H, Viraraghavan T (2003). Heavy metal removal from aqueous solution by fungus *Mucor rouxii*. Water Res. 37: 4486-4496.
- Yilmaz EI (2003). Metal tolerance and biosorption capacity of *Bacillus circulans* strain EBI. Res. Microbiol. 154: 409-415.
- Zalikoff JT, Li JH, Harwig A, Wang XW, Costa M, Rossman TG (1988). Genetic toxicology of lead compounds. Carcinogenesis (Lond.). 9: 1727-1732.
- Zhou P, Thiele DJ (1993). Copper and gene regulation in yeast. Biofactors, 4: 105-115.